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CIRCADIAN FLUCTUATIONS IN TOTAL PROTEIN AND CARBOHYDRATE CONTENT IN THE SLUG *LAEVICAULIS ALTE* (FERUSSAC, 1821)

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Abstract

Rhythmic fluctuations in total protein and carbohydrates in the central nervous system, midgut gland, and foot muscle of *Laevicaulis alte* were not in phase with each other. Fluctuations were largest in the midgut gland. The occurrence of crest values during dark hours for total protein and light hours for total carbohydrates, high protein levels during the dark span and high carbohydrates during light, may be related to the animal's motor functions. Percent loss of water content was bimodal in all three tissues studied.

Introduction

In molluscs, free or combined amino acids form macromolecules, predominantly proteins (Florkin and Bricteux, 1972). Rhythmic fluctuations in various amino acids and proteins over 24-h periods were reported in vertebrates (see Sollberger, 1965) and in the abdominal ganglia of sea hares (Loh and Peterson, 1973). Strumwasser and Wilson (1976) could not detect a rhythm in the formation of protein fractions in the R15 neuron in the abdominal ganglia of *Aplysia*.

Blood sugar levels range widely both within and between species of molluscs, but are generally lower than those of vertebrates (Goddard and Martin, 1966). Depletion of glycogen during starvation in *Cryptozona semirugata* (Ramamurthi and Subramanyam, 1976) is consistent with glycogen use for energy (Meenakshi, 1956). Though there are several studies on cyclical phenomena in carbohydrates among vertebrates (see Sollberger, 1965) such studies on molluscs are scanty.

Physiological rhythms have been found in the acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and acetylcholine (ACh)-AChE systems in the slug *Laevicaulis alte* (Pavan Kumar and Sasira Babu, 1978; Pavan Kumar *et al.*, in press); in ACh, AChE, and electrical activity in the snail *Cryptozona ligulata* (Reddy *et al.*, 1978). The present investigation examines rhythmic fluctuations in carbohydrates and proteins, major sources of energy in the slug *Laevicaulis alte*.

Materials and Methods

Adult, 6–10 gm slugs (*Laevicaulis alte*) were collected during September 1975 from fields around Tirupati (India) and acclimated to laboratory conditions for 10 days in large wooden boxes containing wet mud. Ambient temperature was maintained at 26 ± 2°C and relative humidity 85 ± 5% under a 12:12 (0600 to 1800:1800 to 0600 h) light:dark (L:D) regimen. Ambient temperature and relative

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Abbreviations: CNS, central nervous system; L:D, light:dark ratio; φ, acrophase.

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humidity in the fields where animals were collected were not determined. The animals were fed fresh leaves daily *ad libitum*. (Feeding always coincided with the nocturnal active phase of locomotor rhythm; Pavan Kumar and Sasira Babu, 1979.) Since the slugs are hermaphrodites, sex does not influence rhythmic patterns.

Isolation of tissues and experimental procedure

For experimentation, the 24-h day was divided into six 4-h periods beginning at 0800, 1200, 1600, 2000, 0000, and 0400 h. For 4 days during September 1975, central nervous system (CNS), midgut gland, and foot muscle taken from at least three animals at each period were pooled. The isolated tissues were cleared of body fluids and stored in prechilled glass tubes until analyzed.

Estimate of protein content

Total protein levels in the tissue homogenates were estimated following the method of Lowry *et al.* (1951). Protein content was expressed as micrograms/milligram wet weight of fresh tissue.

Estimate of carbohydrate content

Total carbohydrate levels in tissue homogenates were estimated following the method of Carroll *et al.* (1956).

Tissue homogenates of 1% (weight: volume) were made in 10% trichloroacetic acid (TCA) solution. To 0.5 ml of the centrifuged (3000 rpm for 15 min) clear supernatant, 5.0 ml of anthrone reagent was added and the combination boiled for 10 min in a water bath. The tubes, with their contents, then were immediately cooled. A standard sample containing a known quantity of analar glucose solution was always run along with the experimental samples. The color was measured at 620 nm in Bausch and Lomb Spectronic 20 against a reagent blank. The level of the content was expressed as milligrams/gram wet weight of fresh tissue.

Estimate of water content in the tissues

The CNS, midgut gland, and foot muscle from the slugs were isolated and collected into tinfoil planchets whose empty weights had been previously determined. After the tissues were weighed, they were placed at 100°C in a hot air oven. Weights were determined every 24 h until two readings coincided. Percent loss of water was calculated from the differences in the tissue weights.

Analysis of results

Total protein and carbohydrate content were analyzed for significant differences between data points in a cycle and significant differences between crests and troughs, using Student's t test.

The single cosinor method of Koukkari *et al.* (1974) also was applied to characterize rhythms. Amplitude values were calculated through trigonometric functions and their significance determined through F values obtained. Significance or lack of it at a given level (here $p = 0.05$) is determined based on whether the calculated F value is greater (significant) or less (not significant) than a corresponding value from the F table.

Since at 0000 h the protein content in the CNS exhibited no statistical signif-

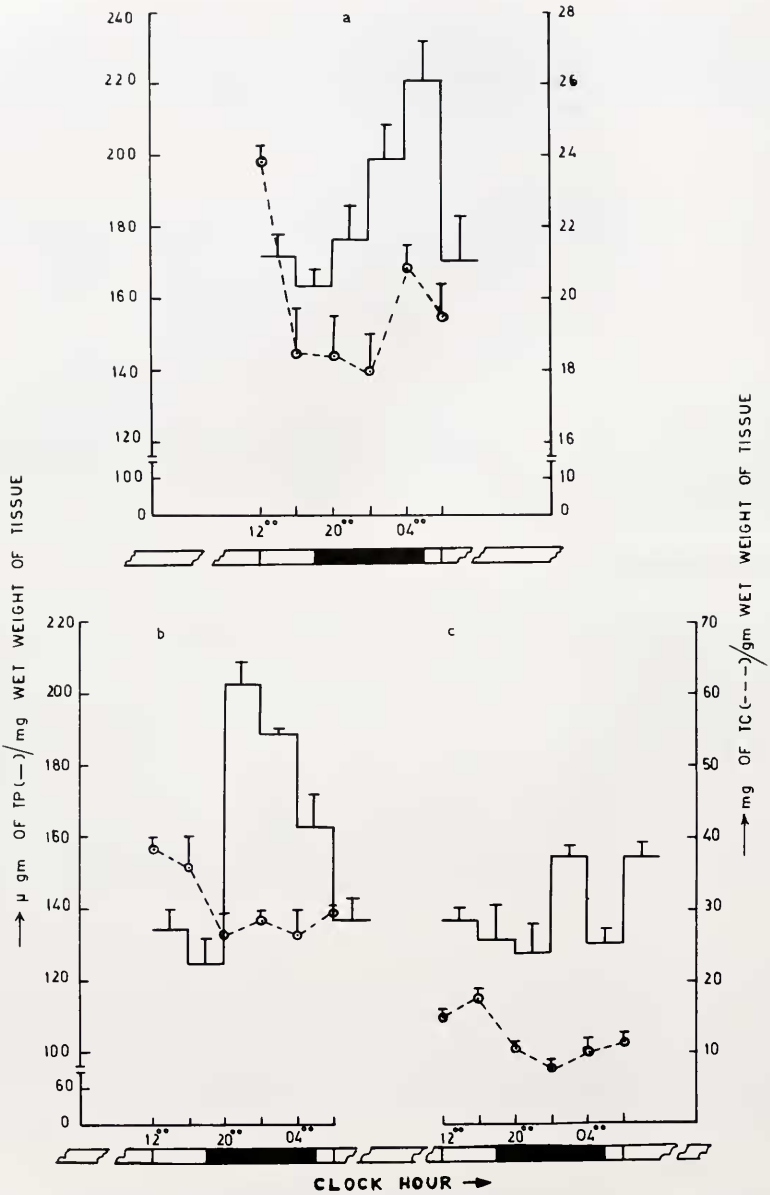

FIGURE 1. Cyclical fluctuations as a function of time in total protein (solid line) and carbohydrate (broken line) contents in the CNS (a, above), midgut gland (b, below left) and foot muscle (c, below right) of *Laevicaulis alte* at L:D 12:12 (0600 to 1800:1800 to 0600 h). Protein content expressed as μgm/mg wet weight of fresh tissue and carbohydrate content as mg/gm wet weight of fresh tissue. Vertical lines = standard error. CNS = central nervous system; MG = midgut gland; FM = foot muscle; Open block = light and bold block = dark hours of 24 h day; TC = total carbohydrate; TP = total protein.

icance for amplitude values, the next time period in the scale (0400 h, when the amplitude was significant) was selected to test amplitude.

The 24 h day was arbitrarily divided into two spans: 0800, 1200, and 1600 h,

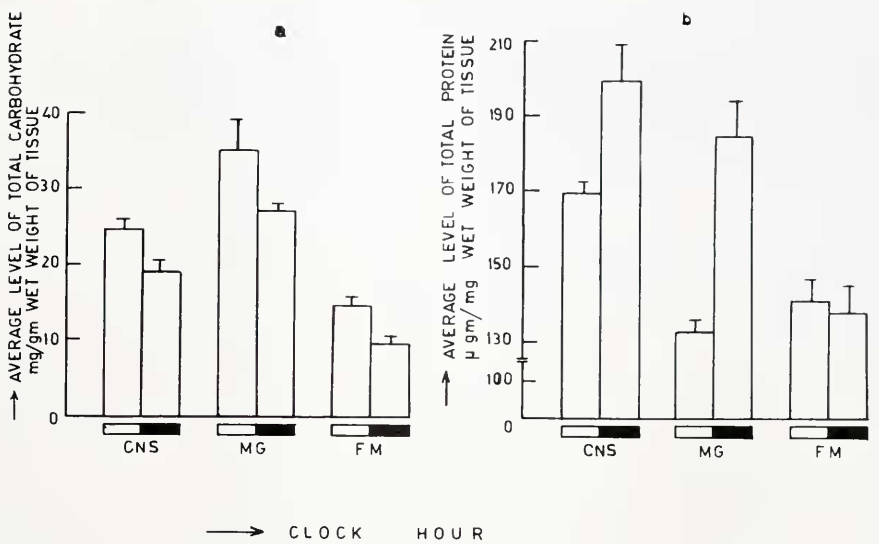

FIGURE 2. Average levels of total carbohydrate (a, left) and protein (b, right) in CNS, midgut gland, and foot muscle of *Laevicaulis alte* during light (0800 to 1600 h, open block) and dark (2000 to 0400 h, solid block) spans of 24 h solar day. CNS = central nervous system; MG = midgut gland; FM = foot muscle.

occurring during light hours (the light span) and 2000, 0000, and 0400 h, occurring during dark hours (the dark span). The averages during the two spans were plotted as histograms (Fig. 2). Percent differences between the average values for light and dark spans were calculated (Table II).

RESULTS

CNS protein and carbohydrate levels

Total protein and carbohydrate levels in the CNS crested at 0400 and 1200 h, respectively (Fig. 1a). Total protein was high during the dark span (Fig. 2b) and carbohydrates high during the light span (Fig. 2a).

Computed crests at −346° for total protein and −154° for total carbohydrate levels (Table I) show that the calculated crest values were closely synchronized with the visual crests in chronograms. (Converted to time, the computed crests were at 0304 h for total protein and at 1016 h for total carbohydrates). While the amplitude values in total carbohydrate content were not significant, they were significant for total protein content (Table I).

Protein and carbohydrate levels in midgut gland

Midgut gland values peaked at 1200 and 2000 h for carbohydrates and proteins respectively (Fig. 1b). As in the CNS, the average values were higher during light for carbohydrates (Fig. 2a) and dark for proteins (Fig. 2b). Computed acrophase (φ, lag from beginning time to the peak) values were synchronized with chronogram crests (Table I). Amplitude values were significant for both protein and carbohydrate (Table I).

TABLE I

Rhythm characteristics M (mesor), A (amplitude), significance, and φ (computed acrophase) for total protein and carbohydrate content in nervous system (CNS), midgut gland (MG) and foot muscle (FM) of Laevicaulis alte *under L:D 12:12 regimen. Amplitude values significant (P = 0.05) when F(2,3) > 9.6. Not significant when F(2,3) (P = 0.05) < 9.6. * Reference local time 0400 h (otherwise 0000 h).*

Source of variables estimated	M ± SD	A ± SD	A Significant/ not significant (S, NS)	φ (range)
TOTAL PROTEIN				
CNS	184.27 ± 19.97	26.71 ± 6.02	S (12.18)	−346°* (−319.5° to 348.5°)
MG	158.04 ± 28.97	41.56 ± 9.08	S (17.88)	−1° (−353° to −9.5°)
FM	139.87 ± 11.38	4.5 ± 2.5	NS (0.13)	−67.5° (−52.5° to −83°)
TOTAL CARBOHYDRATES				
CNS	19.86 ± 2.04	2.09 ± 0.08	NS (1.64)	−154° (−138° to −177°)
MG	31.01 ± 4.67	6.22 ± 2.6	S (11.79)	−203.5° (−184.5° to −230°)
FM	12.08 ± 3.19	4.32 ± 2.5	S (14.96)	−206.5° (−177.5° to −223°)

Protein and carbohydrate levels in foot muscle

In foot muscle, total protein content in the chronogram crested at 0000 and 0800 h (Fig. 1c). But the calculated crest occurred at 0430 h ($\varphi = -67.5°$) (Table I). As in midgut gland, the crest in total carbohydrate content in foot muscle at 1600 h (Fig. 1c) is comparable to a calculated crest at 1346 h ($\varphi = -206.5°$) (Table I).

Protein content in foot muscle did not fluctuate significantly from light to dark spans and carbohydrate levels were large during light (Fig. 2).

Water content in CNS, midgut gland, and foot muscle

In all the tissues, percent loss of water content was bimodal. CNS exhibited the maximum loss and midgut gland the least (Fig. 3).

Other findings

The differences between maximum and minimum values of physiological variables were statistically highly significant at $P < 0.001$ for total carbohydrates in CNS and foot muscle and for total protein in CNS and midgut gland. Differences between high and low values of total carbohydrates in midgut gland and total protein in foot muscle were significant at $P < 0.01$. On overview, carbohydrates, with crests (Figs. 1a to 1c) and higher average values during the light span (Fig. 2), and proteins with the opposite pattern, exhibited uniformity for all the tissues despite discrepancies in the "times" of the crests. Midgut gland registered the

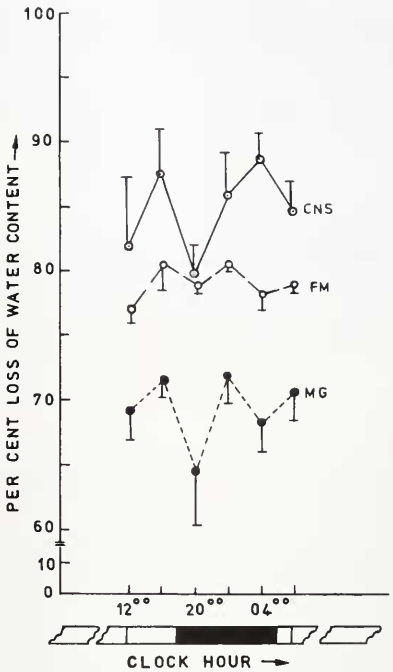

FIGURE 3. Percent loss of water content in CNS, midgut gland, and foot muscle of *Laevicaulis alte* during 24 h L:D regimen of solar day. Vertical lines = standard error. CNS = central nervous system; MG = midgut gland; FM = foot muscle.

highest amplitude for both the variables (Table I). When percent gain and loss from light to dark span and *vice versa* were compared for the variables in the tissues, total protein content increased from light to dark span and an opposite trend was observed for total carbohydrate (Table II; Fig. 2).

DISCUSSION

Laevicaulis alte exhibits a monophasic pattern of motor rhythm, with its active phase during the dark hours of a 24 h day (Pavan Kumar and Sasira Babu, 1979).

TABLE II

Comparison of percent differences in the physiological variables in central nervous system (CNS), midgut gland (MG) and foot muscle (FM) of Laevicaulis alte *under L:D 12:12. + = increment; − = decrement.*

Source of the physiological variable	Light to dark span	Dark to light span
TOTAL PROTEIN		
CNS	+17.95	−15.22
MG	+38.54	−27.82
FM	−2.19	+2.24
TOTAL CARBOHYDRATE		
CNS	−6.64	+7.11
MG	−21.73	+27.75
FM	−34.85	+53.48

This is coupled to cyclical fluctuations in physiological variables such as heartbeat (Pavan Kumar and Sasira Babu, 1976), AChE and BuChE levels (Pavan Kumar and Sasira Babu, 1978), and ACh-AChE system (Pavan Kumar *et al.*, in press), which all crest during the active phase. Similar phenomena were observed in snails (Reddy *et al.*, 1978) and calotes and mice (Pavan Kumar *et al.*, 1979). In the present investigation, an inverse relationship was observed in fluctuations of total protein and carbohydrate levels. Similar inverse relationships have been found in the rates of heartbeat and pause in the slug (Pavan Kumar and Sasira Babu, 1976), 5-hydroxytryptamine content in different parts of the brain of turtles (Quay, 1967), and glycogen content in mice (Barnum *et al.*, 1958). The opposition of total protein to the rhythmic pattern of total carbohydrate content suggests that carbohydrates accumulate during the rest period. Accumulation from dietary sources would account for higher levels of carbohydrates during the light span, since the active phase of motor rhythm and the feeding of the animal coincide (Pavan Kumar and Sasira Babu, 1979).

The amplitude values (Table I) for protein and carbohydrate in the midgut gland clearly document that the fluctuations, and thus the turnover of the constituents, are larger in this gland than in other tissues examined. The fluctuations of the constituents in this organ clearly are statistically significant. Acceptance of the null hypothesis for total carbohydrates in CNS and total protein in foot muscle, reflecting no significant amplitude values, suggests a different situation. In the CNS carbohydrates probably are used for energy while in foot muscle proteins may meet requirements for other associated reactions. This assumption is supported by the small differences in percent loss and gain for total carbohydrates in CNS and total proteins in foot muscle (Table II).

The cresting and high average levels in total protein content during dark hours suggest that use or turnover of protein (and possibly associated reactions) may be high during the active phase of motor activity in *Laevicaulis*. Protein use (Meenakshi, 1956) is associated with higher levels of certain enzymes in aestivating *Pila globosa* (Murthy *et al.*, 1974). The transamination reactions in slugs and cockroaches (Pavan Kumar, 1976; Pavan Kumar and Sasira Babu, in press; Sasira Babu *et al.*, 1977; Vijayalakshmi *et al.*, 1978) are high during the animals' active phases of motor rhythm.

The cresting and high average levels of the total protein content during dark periods in all three tissues suggests that protein synthesis might be high during the inactive phase, since accumulation requires time. Protein formation might be high during active phase. Loh and Peterson (1973) observed that the 12K protein fraction in the abdominal ganglia of *Aplysia* is high at dawn. This is in association with the circadian motor pattern (Jacklet, 1972). However, Strumwasser and Wilson (1976) could not detect a circadian pattern in formation of protein in the abdominal ganglion of *Aplysia*. It also has been suggested that the time of dissection has a bearing on the physiological processes of the ganglion (Audesirk and Strumwasser, 1975).

Circadian rhythms in carbohydrates in different groups of animals have been reviewed (see Sollberger, 1965). Among invertebrates, circadian rhythms occur in carbohydrates in crustaceans (Dean and Vernberg, 1965) and insects (Nowosielski and Patton, 1964). Relationship of carbohydrates to energy source in *Laevicaulis alte* is reflected in krebs cycle enzymes (succinate dehydrogenase, Pavan Kumar, 1976; Pavan Kumar and Sasira Babu, in press a) and associated enzymes like aspartate and alanine aminotransferases (Pavan Kumar, 1976; Pavan Kumar and Sasira Babu, in press b). Thus, high total carbohydrate levels during the inactive

phase might result from the need to meet energy requirements for synthesis of various biological constituents while decreased levels during the active phase reflect utilization of carbohydrates for motor functions that demand energy. In conformity with this, the observed fluctuations in total carbohydrate content were greatest in foot muscle (Table II), which is actively engaged in locomotion.

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